IV. ENZYMES

On Enzymic and Chemical Reactions in Crushed Plants

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Some enzymic and chemical reactions, which are characteristic of many plants when they are crushed, are described mostly on the basis of the investigations performed during some years in the Biochemical Institute, Helsinki. Special attention has been fixed on the original plant substances which function as substrates in the enzymic reactions and which are the precursors of many biologically interesting substances, e.g., antimicrobial substances, growth hormones, and antithyroid compounds, not to be found in intact plants. The question of how benzyl thiocyanate is formed in the seeds of *Lepidium sativum*, a reaction demonstrated some years ago in this laboratory, has been treated in greater detail. The existence of a new enzyme, isothiocyanate isomerase, has been demonstrated.

My article in this jubilee volume on the biochemistry of large molecules dedicated to the sixtieth birthday of Arne Tiselius deals more with low molecular compounds than with large molecules in plants. The peculiar situation that some enzymes and their substrates are separated from each other in intact plants and come into contact with each other only when the plants are crushed or otherwise injured is characteristic of plants. As a consequence of this, the enzymic splitting of low molecular precursors when man eats vegetables, or domestic animals fodder plants, is a remarkable process. If the product formed by the enzymic reaction is unstable, as happens fairly often, a series of chemical reactions may follow the primary enzymic reaction. Enzymic and chemical reactions are then so entangled that it is impossible to understand how the different products are formed without knowing the structure of the precursors and their enzymic cleavage. The recent finding in this laboratory that different products can be formed from the same substrate, depending on the plant species, raises new questions concerning the relations between enzymes and substrates in plants. This, I think, justifies the inclusion of this article with its stress on organic chemical material in the present volume.

When investigating substances in cabbage that interfere with the function of the thyroid gland, we had to solve the question of how the thiocyanate ion (SCN^-), which inhibits the uptake of iodide in the thyroid gland, is formed in cabbage. The presence of SCN^- in cabbage was observed some years ago by Michajlovskij and Langer (1).

In investigations performed in this laboratory the presence of SCN^- could not be established in intact cabbage, but a rapid and profuse formation of SCN^- was observed when cabbage was crushed; hence animals and man will really obtain SCN^- when cabbage is used as food (2). When the origin of SCN^- was further investigated, its precursor was isolated from cabbage as a crystalline tetramethylammonium compound and proved to be a thioglucoside of a new type, containing an indole group (3). On the basis of the enzymic, hydrolytic, and hydrogenolytic decomposition products, Gmelin and I (4, 5) gave the formula presented in Fig. 1 (Structure I) to the glucoside, which we called glucobrassicin.

The isolation of glucobrassicin explained

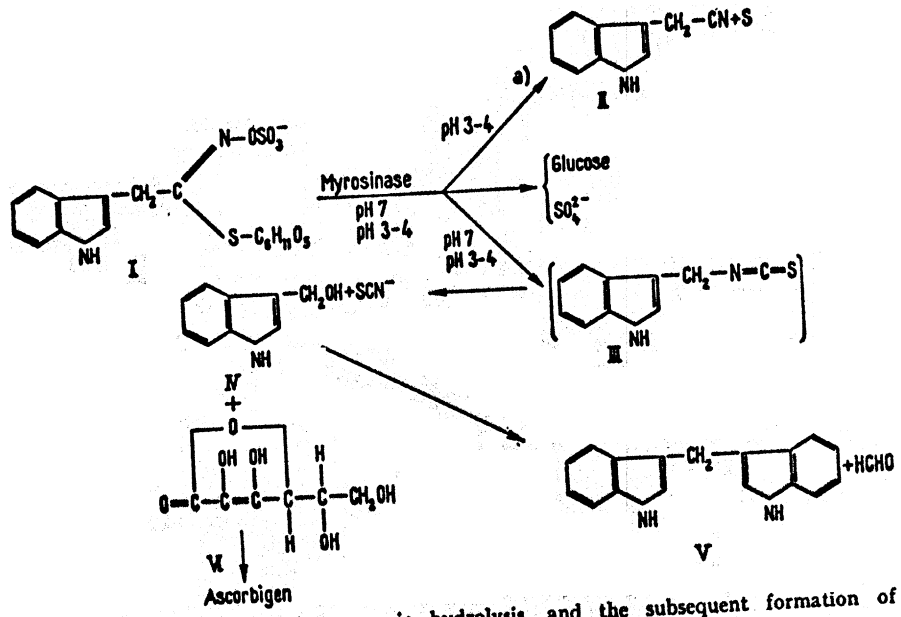

FIG. 1. Glucobrassicin, its enzymic hydrolysis, and the subsequent formation of different products.

the formation of SCN^- as well as that of many other compounds that had earlier been regarded as original plant substances.

According to the scheme shown in Fig. 1, the isothiocyanate (III) which should be formed from glucobrassicin, in analogy with other mustard oil glucosides, is so unstable because of its indole group that it is immediately split into SCN^- and 3-hydroxymethylindole (IV). The formation of SCN^- is quantitative at a neutral reaction and makes it possible to determine glucobrassicin quantitatively in cabbage plants. Because some *Brassica* plants contain smaller amounts of N_1-methylglucobrassicin too (6), the SCN^- formed from these plants corresponds to both thioglucosides. When cabbage is boiled, SCN^- is also formed, but only half the amount formed enzymically (5). This is due to the fact that glucobrassicin is then decomposed in two different ways.

Ascorbigen is another substance formed in crushed cabbage. As early as in the 1930's, Guha and Pal (7) found that "bound ascorbic acid," which releases ascorbic acid on hydrolysis, can be extracted from cabbage with organic solvents. These authors gave the name ascorbigen to the substance although they did not isolate it. Procházka, Šanda, and Šorm (8) separated it from other substances. The compound was amorphous but formed crystalline derivatives, e.g., with picric acid.

Gmelin and I (5) found that ascorbigen can be synthesized either from 3-hydroxymethylindole and ascorbic acid or from indole, formaldehyde, and ascorbic acid simply by heating the aqueous suspensions of these substances. According to later experiments (9), the synthesis is rapid even at room temperature in an acid reaction. The optimal pH for the synthesis is between 3 and 5 (Fig. 2).

On the basis of the structure of glucobrassicin and the syntheses of ascorbigen, it is apparent that ascorbigen is formed in cabbage from the 3-hydroxymethylindole arising as a decomposition product of glucobrassicin and the ascorbic acid always present in cabbage. Ascorbigen is thus no native plant substance at all, but a compound formed secondarily when cabbage is crushed. The structure of ascorbigen presented by Procházka *et al.* (8) has to be revised to comply with the synthesis of ascorbigen from 3-hydroxymethylindole and ascorbic acid. The structure of ascorbigen is not yet known in detail, but an ether bridge between the two components is probable.

Figure 1 also shows that 3-indoleaceto-

be prevented, and the isolation of original plant substances is made possible. But even now great difficulties may occur if the substance is so unstable that it is changed in some way or decomposed even when the enzymes are destroyed. In addition, the isolation of precursors in plants, e.g., of the glucosides, is often much more difficult than that of the aglucones. In any case, even in recent years the literature has contained numerous pieces of information about substances which in reality do not exist in plants.

When the nutrition of man and animals is involved, the secondary substances are usually the most interesting ones because of their biological activity. When man eats vegetables, for instance, or the cow fodder, the precursors present in plants are decomposed by the influence of plant enzymes and the secondary substances pass into the organism (35). On the basis of the above, fresh and cooked vegetables differ essentially from each other.

ACKNOWLEDGMENT

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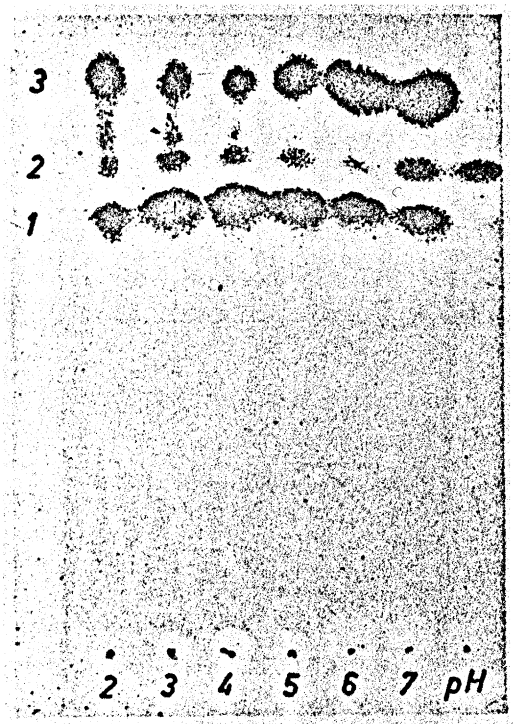

FIG. 2. Synthesis of ascorbigen from 3-hydroxymethylindole and ascorbic acid in water at 37° C. and different pH values. A paper chromatogram of the products formed: (1) ascorbigen, (2) 3-hydroxymethylindole, (3) diindolylmethane. Reaction time 10 min. Control with 3-hydroxymethylindole to the extreme right.

nitrile (II), a strong growth hormone in plants, is formed at an acid reaction from glucobrassicin after the enzymic splitting off of glucose and sulfate. The splitting off of sulfur, resulting in indoleacetonitrile, is a nonenzymic reaction. When glucobrassicin was boiled in aqueous solution, considerable amounts of 3-indoleacetonitrile were also formed. On acid and alkaline hydrolysis 3-indoleacetic acid too was formed, besides other indole derivatives (5).

Many investigators had previously come to the conclusion that cabbage plants contain large amounts of bound growth hormones (10). Linser *et al.* (11) have paid special attention to the unusually high content of bound 3-indoleacetonitrile in *Brassica* leaves and considered it possible that the strange form and size of the leaves of many *Brassica* plants could be connected with this accumulation of bound growth hormones. Because the bound hormone had no growth-promoting effect, it was thought that the hormone was released when needed. That was a tempting hypothesis which could have made the peculiar forms of cabbage plants understandable if it had been possible to prove it experimentally.

The discovery of glucobrassicin in cabbage plants, and the formation of considerable amounts of 3-indoleacetonitrile from it at low pH, give an explanation to the earlier findings. There is in fact no bound growth hormone in *Brassica* leaves, but 3-indoleacetonitrile and also 3-indoleacetic acid are formed from glucobrassicin when the extraction methods of earlier authors are used. Glucobrassicin has no growth-promoting effect in the *Avena* coleoptile test, whereas the hydrolyzates of glucobrassicin show a high effect (5).

In spite of the chemical explanation of the formation of growth hormones when cabbage plants are crushed, extracted, or boiled at low pH, the physiological significance of glucobrassicin as the precursor of these hormones in intact plants is still unknown. Glucobrassicin and the enzyme complex, myrosinase, which is responsible for the cleavage of the thioglucoside, presumably occur in different cells in plants, and come into contact with each other only after the crushing of cells. The possibility that these components should reach each other in the growing point, where the division of cells is rapid, may exist, but at least at the present moment we have no experimental proof for it.

Cabbage plants are good examples of how difficult it is to avoid erroneous conclusions in regard to substances isolated from plants, if the original precursors in the plants and their enzymic and chemical cleavage products are not known.

Concerning the enzymic cleavage of mustard oil glucosides, the remarkable discovery was made by Gmelin and me (12) that in the crushed and moistened seeds, as well as in the green plants, of *Lepidium sativum* and *ruderale*, benzyl thiocyanate (BTC) was formed in addition to smaller amounts of benzyl isothiocyanate (BITC), or alone. This was the first time a thiocyanic acid ester was found in nature. In corresponding experiments with *Tropaeolum majus*, which con-

tains the same benzyl thioglucoside, glucotropaeolin, as *Lepidium*, only BITC was formed. Gaines and Goering (13, 14) demonstrated that "myrosinase" is composed of two enzymes, one of which splits off glucose and the other sulfate. Ettlinger *et al.* (15) came to a similar conclusion and showed that one of the enzymes is the classical myrosin(ase). The other enzyme requires vitamin C as a cofactor. Nagashima and Uchiyama (16) had earlier observed that myrosinase was strongly activated by ascorbic acid. One could therefore think that the reason for a different rearrangement of the same substrate in *Lepidium* and *Tropaeolum* could be traced back to differences in the relative activity of both enzymes in these plants. This hypothesis does not, however, give a satisfactory explanation to the problem, as will be seen from the results presented below.

Saarivirta and I (17) have studied the peculiar difference between *Lepidium* and *Tropaeolum* regarding the enzymic cleavage products of glucotropaeolin. The paper chromatograph finding that glucotropaeolin alone was present in the seeds of *Lepidium*, and that both BTC and BITC were accordingly formed from this thioglucoside, had at first to be confirmed. We found that the addition of citrate buffer instead of water to crushed *Lepidium* seeds strongly increased the formation of BITC and correspondingly decreased that of BTC (Table I). When water was added to the crushed seeds at room temperature, the ratio of BTC to BITC was about 2.5. The addition of 0.5 *M* citrate buffer instead of water reversed the ratio to 0.25. The total amount of both compounds was about the same independently of the variations of their mutual ratio.

This is a decisive proof of the formation of BTC and BITC in *Lepidium* seeds from the same thioglucoside, glucotropaeolin, from which only BITC is formed in *Tropaeolum* seeds. It was further observed that the addition of water or citrate buffer solution to

TABLE I

FORMATION OF BENZYL THIOCYANATE (BTC) AND BENZYL ISOTHIOCYANATE (BITC) IN CRUSHED *Lepidium sativum* SEEDS WHEN WATER OR DIFFERENT BUFFER AND SALT SOLUTIONS WERE ADDED TO THE CRUSHED MATERIAL

Temperature approx. 22° C., time 15 min., pH 5–5.2[a]

Additions	mg./100 mg. seeds			
	BTC	BITC	BTC+BITC	BTC/BITC
Water	728	310	1038	2.35
Citrate buffer				
0.3 *M*	268	806	1078	0.33
0.5 *M*	224	814	1038	0.25
1.0 *M*	216	799	1015	0.27
Acetate buffer				
0.1 *M*	564	446	1010	1.27
0.5 *M*	436	595	1034	0.73
NaCl				
1.75%	568	396	964	1.43
5.80%	252	432	684	0.58
16.80%	140	396	536	0.35
22.70%	80	360	420	0.22
KCl				
2.24%	428	461	889	0.93
7.50%	300	403	703	0.74
22.70%	88	418	506	0.21
Na_2SO_4, 14.20%	444	274	718	1.62
Water, heated seeds[b]	0	1360		

[a] Variations in the acidity between pH 4.6 and 6.6 did not much influence the amount of BTC.

[b] Seeds were heated at 100° C. for 5 hr. to destroy the enzymes. A crude "myrosinase" extract from *Lepidium sativum* was added to the inactivated, crushed material.

the crushed seeds of *Tropaeolum* brought about the formation of BITC only.

The following observations also elucidate the effect of different factors on the formation of BTC and BITC (Table I).

Neutral salts (sodium and potassium chloride) strongly reduce the ratio of BTC to BITC. The salt effect increases when the concentration rises. The influence of citrate buffer, too, depends on the salt effect. The pH of the solution has no greater effect on the reaction products in the range of 4.6 to 6.2.

The results were not easy to understand. It was first believed that glucotropaeolin in *Lepidium* seeds is bound with a basic group of some protein and that the rearrangement after the splitting of glucose is directed to the sulfur atom. Then we began to study the rate of the reaction and the influence of the temperature on the ratio of BTC to BITC (Fig. 3). The reaction time was 5 min.

The results in Fig. 3 clearly show that the ratio of BTC to BITC rapidly decreases when the temperature rises from 2 to 37° C.

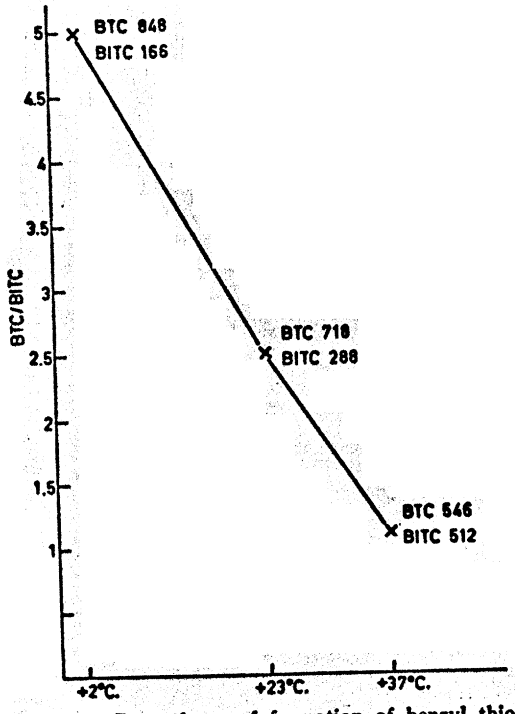

FIG. 3. Dependence of formation of benzyl thiocyanate (BTC) and benzyl isothiocyanate (BITC) in crushed, moistened seeds of *Lepidium sativum* on temperature. Time 5 min.

The rate of the reaction is so high that the reaction was brought to completion in a few minutes even at 2° C. Accordingly, the results in Fig. 3 represent end products of the reaction. When BTC and BITC were estimated after very short times, an entirely different view of the formation of BTC was obtained (Table II).

TABLE II

FORMATION OF BTC AND BITC AT 2° C. WHEN THE ENZYMIC REACTION WAS BROKEN OFF AFTER VERY SHORT REACTION TIMES FOLLOWING ADDITION OF WATER TO CRUSHED SEEDS OF *Lepidium sativum*

Reaction time, sec.[a]	µg./100 mg. seeds			
	BTC	BITC	BTC+ BITC	BTC/ BITC
10–15	188	623	811	0.30
30	456	392	848	1.16
60	672	247	919	2.72
120	736	234	970	3.14
180	800	187	987	4.28
300	848	181	1029	4.69
1800	849	173	1022	4.91

[a] The reaction times under 60 sec. are only approximate.

From the results given in Table II it appears that after a few seconds at 2° C. the ratio of BTC to BITC is < 0.3, but that it rises very rapidly. After 60 sec. it is already 2.7 and, after 5 min., when the reaction is completed, 4.7. The results suggest that the Lossen rearrangement after the enzymic splitting of glucose and sulfate also in crushed, moistened *Lepidium* seeds leads to the formation of BITC. This is, however, rapidly isomerized to BTC. Because BITC is not spontaneously isomerized to BTC (e.g., in crushed, moistened *Tropaeolum* seeds only BITC is formed from glucotropaeolin) the reaction is obviously enzymic. This means that *Lepidium* seeds contain an isothiocyanate isomerase, an enzyme unknown until now. The probable pathway for the formation of benzyl thiocyanate (III) from glucotropaeolin (I) over benzyl isothiocyanate (II) is shown in Fig. 4.

On the basis of the ratio of BTC to BITC at different temperatures, the activity of the

isomerase decreases when the temperature rises from 2 to 37° C. (Fig. 3). The enzyme has not been found in the water extract prepared from *Lepidium* seeds because the addition of this extract to heated, crushed *Lepidium* seeds leads only to the formation of BITC as mentioned above. On the basis of the recent observation that a water extract of the green part of the *Lepidium ruderale* plant brought about a formation of BTC from added glucotropaeolin the isomerase could partly be dissolved from this material, however. Studies of the isomerase are in progress in this laboratory.

Allyl thiocyanate is formed from the allyl thioglucoside, sinigrin, contained in *Thlaspi arvense*, and hence the formation of thiocyanic acid esters is not restricted only to the formation of BTC in *Lepidium*. Judging by the smell, allyl thiocyanate is also formed in some other plants (12).

With young cereal plants we have also made observations which illustrate mistakes that can be made when isolating plant substances. When in the middle of the 1950's work was started in this laboratory to elucidate the cause of the different resistances of several rye varieties to *Fusarium nivale*, Hietala and I (18) isolated a substance in crystalline form with antifungal properties from the ethanol extract of crushed rye seedlings. The substance was found to be benzoxazolinone (BOA) (Fig. 5). Because this substance contained no hydroxyl group, there was no possibility of presuming that it occurred as a glucoside in plants, and so BOA was at first regarded by us as an original plant substance. By analogy, we came to the

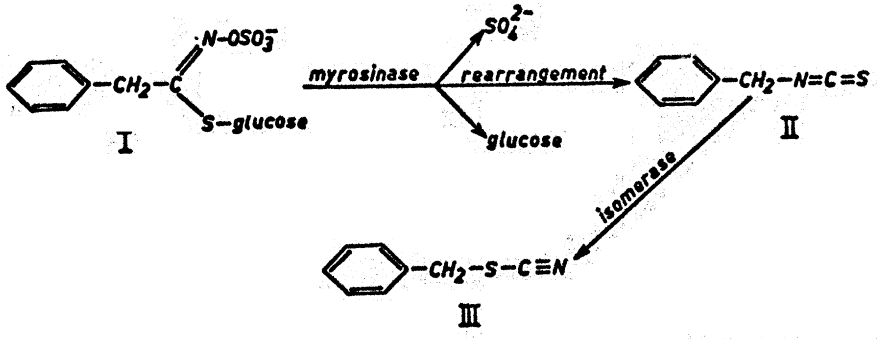

FIG. 4. Pathway for formation of benzyl isothiocyanate (II) and benzyl thiocyanate (III) from glucotropaeolin (I).

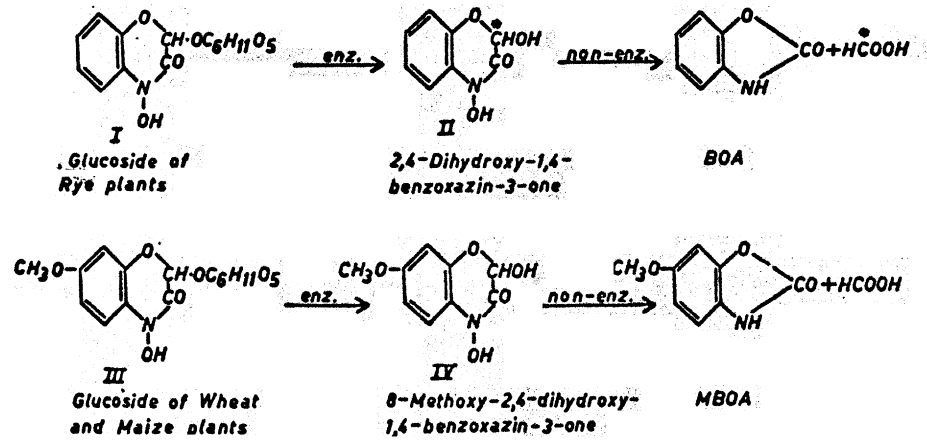

FIG. 5. Glucosides from young rye, wheat, and maize plants, their aglucones, and the formation of benzoxazolinones from the aglucones.

same conclusion in regard to 6-methoxybenzoxazolinone (MBOA), which was isolated from young maize and wheat plants (19). Later observations, however, were difficult to understand on the basis of this conception, and further investigations finally led to the isolation of a new type of substance from these plants, and to the finding of an explanation for the formation of BOA and MBOA (20–23). Figure 5 shows that the precursor of BOA is a glucoside which is enzymically hydrolyzed when the plant is crushed. The aglucone part of the glucoside differs essentially from BOA. In the original aglucone the heterocyclic ring joined to the benzene ring is 6-membered, while in BOA it is 5-membered. The conversion of the aglucone into BOA is an intramolecular redox reaction by which one carbon atom in the heterocyclic ring is oxidized to formic acid. The reaction takes place when the aqueous solution of the aglucone is heated. When the aglucone was prepared synthetically (24), and carbon atom 2 could be labeled with C^{14}, the formic acid which was split off when the 5-membered ring was formed contained all the C^{14} (25). The mechanism and kinetics of the reaction have recently been investigated in detail in this laboratory (26). According to this investigation, the formation of BOA takes place still more readily in ethanolic than in aqueous solution, and that is the reason why we first isolated BOA from rye plants. The glucoside is hydrolyzed not only by an enzyme present in rye, wheat, and maize plants, but also in the wall of the small intestine. Yeast does not hydrolyze the glucoside (25).

BOA and MBOA have a considerable antifungal effect. Since they are not present in the seedlings, they cannot cause the resistance against *Fusarium*. The aglucone formed from the glucoside on hydrolysis also has an antifungal effect. The aglucone does not occur in free form in rye seedlings, however. A problem arises in this connection, similar in principle to that discussed above, concerning the formation of the plant growth hormone, 3-indoleacetonitrile, from glucobrassicin in cabbage. Could the ineffective precursors be of any importance in the growing plants, where they are separated from the corresponding enzymes and the enzymic formation of active substances occurs only after the crushing of the cells? The possibility exists that a hydrolysis of the glucoside could take place when the seedlings are weakly infected by *Fusarium nivale*, but so far we have no experimental proof for this hypothesis. On the other hand, we have observed that substances possessing an antifungal effect are present in intact rye seedlings in which the enzymes have been destroyed by ethanol. So far, we have not succeeded in isolating these substances, but it is possible that they bring about the resistance of the seedlings to fungal diseases.

Yet another class of substances isolated from a plant deserves to be mentioned in this connection. Some years ago, Matikkala and I (27) isolated a sulfoxide from onion, and proved it to be a cyclic compound, 3-methyl-1,4-thiazane-5-carboxylic acid 1-oxide (Fig. 6, V). The structure was confirmed by synthesis (28) and the name cycloalliine was given to the substance, because its elementary composition was the same as that of alliine, and it could be thought to be formed from alliine. The path of formation of this sulfoxide remained unclear for some years. However, a year ago Spåre and I (29) isolated a new sulfoxide from onion which turned out to be the precursor of the lachrymatory substance. This sulfoxide proved to be (+)-*S*-(prop-1-enyl)-L-cysteine *S*-oxide (Fig. 6, I). By the influence of an enzyme isolated from onion, the lachrymatory substance was formed from this compound. The enzyme could be divided into two components on a Sephadex column. Both components decomposed the sulfoxide to the lachrymatory factor, pyruvic acid, and ammonia. It was suggested that the lachrymatory factor could be propenylsulfenic acid (II) (30), and mass spectrometric determinations gave strong support to this idea (31). The lachrymatory factor represents the first aliphatic sulfenic acid known. It is a fairly unstable compound, too, which decomposes to propionaldehyde. Some 2-methylpent-2-enal is formed from it.

The sulfoxide (I) is present in the onion also in a γ-glutamyl peptide in relatively large amounts (32). So far, nine γ-glutamyl peptides have been isolated from the onion (33), γ-L-glutamyl-(+)-*S*-(prop-1-enyl)-L-

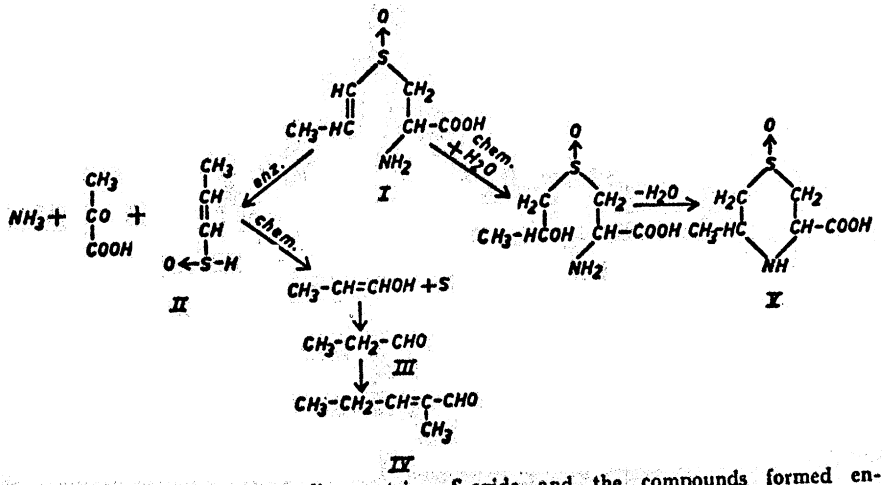

FIG. 6. (+)-*S*-(Prop-1-enyl)-L-cysteine *S*-oxide and the compounds formed enzymically and chemically from it.

cysteine *S*-oxide (peptide 4) being the most important of them quantitatively. The peptide could be hydrolyzed by an enzyme preparation from calf kidney, but strangely enough by no preparations from onion (32). When the green leaves push out from the onion, the γ-glutamyl peptides present in the bulb begin to disappear, which means that they are used in the nitrogen metabolism of the growing onion. It is, however, unclear in what way the peptides are used.

Cycloalliine, too, was found to be formed spontaneously from the precursor of the lachrymatory substance in aqueous solution at room temperature (pH > 7). After this finding, the question arose whether the cycloalliine we had isolated from onion was really an original substance in onion or a compound formed during the isolation process from the lachrymatory precursor. To elucidate this problem, a whole intact onion was heated in 6 *N* HCl, when, according to our earlier determinations, cycloalliine is partly reduced to the corresponding thio ether, and partly oxidized to several compounds, among which methyltaurine and cysteic acid were characterized in connection with the structure determination of cycloalliine (28). The precursor of the lachrymatory substance again is unstable on hydrolysis, even when boiled in 0.5 *N* HCl. Neither is it cyclized in an acid solution. The result of our experiment was that the thio ether of cycloalliine was formed in intact onion on heating with hydrochloric acid. It was thus proved that cycloalliine is present in intact onion (34). A considerable part of it can be formed, however, during the isolation of amino acids from onion, because when amino acids are eluted with 1 *N* ammonia from the Amberlite IR-120 column, and when ammonia is further evaporated from the eluate, the cyclization of the lachrymatory precursor to cycloalliine takes place.

In this case the isolated sulfoxide thus turned out to be an original plant substance, but also a secondary product formed during the isolation process. Before the isolation of the lachrymatory precursor, and before its properties were known, it was impossible to realize that cycloalliine could be formed during the isolation process.

The examples presented above illustrate how numerous and complicated chemical reactions may be evoked by one enzymic reaction in plants, and how difficult it may be, when isolating new substances from plants, to know if one has to do with an original substance or a secondary product not present in the plant as such. In simple cases, for instance when stable phenolic compounds are in question, which occur in plants either bound to sugars as glucosides or free or partly bound, partly free, it is easy to recognize an original plant substance. The situation is far more complicated, however, in the instances I have given. By destroying the enzymes in intact plants, enzymic reactions can